5. On account of the variable and low results obtained with clotted blood, it may be inferred that the method of Nicloux may give low results when applied to organs or tissues which do not disintegrate when boiled with acid alcohol. We take this opportunity of stating that the expenses of the foregoing work were defrayed out of a grant made by the Royal Society.

Cyanogenesis in Plants. Part VI.—On Phaseolunatin and the Associated Enzymes in Flax, Cassava, and the "Lima Bean."

By Professor WYNDHAM R. DUNSTAN, M.A., LL.D., F.R.S., T. A. HENRY, D.Sc. (Lond.), and S. J. M. AULD, Ph.D. (Würzburg).

(Received February 27,—Read February 28, 1907.)

In a previous paper of this series* it has been shown that the production of prussic acid by the seeds or beans ("Lima beans") of *Phaseolus lunatus* is due to the interaction of a cyanogenetic glucoside, phaseolunatin, with an enzyme, both these substances being proved to exist in the seeds.

Phaseolunatin was proved to have the composition and constitution of a dextrose ether of acetonecyanohydrin, but it was not then obtained in sufficient quantity to ascertain precisely the structure of the dextrose residue in the glucoside. Recently, however,† large supplies of "Java beans" (the beans produced by *Phaseolus lunatus* grown in Java) have been imported into this country, and we are indebted to Dr. Bernard Dyer for a small consignment of these beans, which has constituted the raw material from which the considerable quantities of phaseolunatin required in the course of the present investigation have been prepared.

Since the publication of our previous paper, it has been asserted by Kohn-Abrest‡ that these "Java beans" contain not one, but several cyanogenetic glucosides, and that none of these yield acetone on hydrolysis by hot dilute mineral acids or by the glucosidolytic enzymes present in the beans.

We have considered it necessary, therefore, to examine carefully the glucosidic product obtained from "Java beans" by the process originally used by us§ in the investigation of the beans of *Phaseolus lunatus* obtained from Mauritius and we have been unable to detect the presence of any other cyanogenetic glucoside in Java beans except phaseolunatin, identical in all

* Dunstan and Henry, 'Roy. Soc. Proc.,' 1903, vol. 72, p. 285.

† 'Bulletin of the Imperial Institute,' 1905, vol. 3, p. 373, and 1906, vol. 4, p. 329.

‡ 'Comptes rendus,' 1906, vol. 143, p. 182.

§ Dunstan and Henry, *loc. cit.*

respects with that first isolated by us from the practically wild *Phaseolus lunatus* of Mauritius.* We have found no difficulty in confirming our previous observation that this glucoside yields acetone on hydrolysis, and is, in fact, a dextrose ether of acetonecyanohydrin.

We have shown recently† that the linamarin first isolated by Jorissen and Hairs from flax (linseed) is identical with phaseolunatin, and that the latter also occurs in the cassava of tropical countries. This identification of linamarin with phaseolunatin makes it possible to discuss the divergent views, which have been held by various investigators regarding the nature of the enzyme, which occurs in association with phaseolunatin in flax and in the beans of *Phaseolus lunatus*.

Jorissen, in his first paper‡ dealing with the production of prussic acid by flax, stated that the seed contained a substance on which emulsin acted in the same way as on amygdalin. In a later paper, Jorissen and Hairs§ recorded the isolation of linamarin (phaseolunatin) from flax and stated that it was decomposed by the enzyme associated with it in flax seed, but not by the emulsin of almonds. Subsequently linamarin was re-examined by Jouck,‖ who stated that it was decomposed by emulsin. In the third paper of the present series,¶ dealing with the isolation of phaseolunatin, the identity of which with linamarin had not then been established, it was stated that this glucoside was decomposed by emulsin, and that as the range of activity of the enzyme, occurring with phaseolunatin in the beans of *Phaseolus lunatus*, appeared to be identical with that of the emulsin of almonds, it might be assumed provisionally that these beans contained emulsin.

This statement was based on the result of a single experiment, in which prussic acid was undoubtedly liberated when a commercial emulsin preparation was added to an aqueous solution of the glucoside. This experiment has, however, been repeated frequently, using several different commercial emulsin preparations as well as emulsin prepared by ourselves from sweet almonds, and in no case have we been able to observe again the formation of prussic acid within a reasonable time, though in every case prussic acid was liberated on the further addition of the mixture of enzymes prepared from the beans of *Phaseolus lunatus* or from flax or cassava.

* M. Kohn-Abrest's results are dealt with more fully in a recent paper (Dunstan and Henry, 'Annales de Chimie et de Physique,' January, 1907).

† Dunstan, Henry, and Auld, 'Roy. Soc. Proc.,' 1906, vol. 78, p. 145.

‡ Jorissen, 'Bull. Acad. Roy. Belg.,' 1884 (iii), vol. 6, p. 718.

§ Jorissen and Hairs, 'Bull. Acad. Roy. Belg.' (iii), vol. 21, p. 529.

‖ Jouck, 'Beiträge zur Kenntnis der Blausäure abspaltenden Glycoside,' Strassburg, 1902.

¶ Dunstan and Henry, 'Roy. Soc. Proc.,' 1903, vol. 72, p. 285.

The statement that phaseolunatin is decomposed by the emulsin of almonds must therefore be withdrawn. It is probable that in the cases (recorded by Jorissen,* Jouck,† and in the third paper of this series‡) in which the liberation of prussic acid from phaseolunatin has been attributed to the action of emulsin the decomposition was in reality due to some secondary cause and not to the direct agency of emulsin.

There is, therefore, the fundamental difference between the emulsin of almonds and the enzymes occurring in *Phaseolus lunatus* beans, flax, and cassava, that whilst emulsin decomposes amygdalin and salicin and is without action on phaseolunatin, the enzymes of *Phaseolus lunatus*, flax, and cassava decompose all three of these glucosides. The question at once arises as to whether the enzyme associated with phaseolunatin in these three plants is merely an enzyme of the emulsin type having a greater range of activity, or is a mixture of emulsin with a second enzyme, and that the decomposition of phaseolunatin is due to the action of the latter. The first of these hypotheses seems at first sight the more plausible, since it is difficult to admit that the same mixture of two enzymes should occur in the three plants already mentioned, especially since none of them contains, so far as has been ascertained, any glucoside or complex sugar which could be attacked by emulsin. The further evidence we have obtained, however, leaves no doubt that, accepting present theories of enzyme action, these three plants do contain two enzymes, one of the emulsin type and the other of the maltase type.§ It is probable that to the action of the "maltase" the decomposition of phaseolunatin is due.

Fischer‖ has shown that the glucosidolytic enzymes so far systematically examined are divisible into two classes, the one capable of decomposing the α-alkyl ethers of the hexoses and the other the stereo-isomeric β-alkyl ethers of these sugars. The first class of enzymes may be regarded as typified by the maltase of yeast and the second by the emulsin of almonds.

It is obviously possible from Fischer's generalisation to classify an

* Jorissen, *loc. cit.*

† Jouck, *loc. cit.*

‡ Dunstan and Henry, *loc. cit.*

§ Since the present investigation was concluded, some results have been published by Marino and Fiorentino ('Gaz. Chim. Ital.,' 1906, vol. 36, p. 395), which seem to indicate that the maltase of malt is capable of decomposing both α- and β-glucosides; thus, according to these authors, it effects the decomposition both of maltose and salicin. They reject the obvious and simple explanation of these anomalous results, viz., that the maltase they used contained emulsin, on what appear to be insufficient grounds. If their conclusions were established, our present views respecting the nature and mode of action of the group of glucosidolytic enzymes would require to be considerably modified.

‖ 'Zeit. Physiol. Chem.,' 1898, vol. 26, p. 75.

unknown glucosidolytic enzyme by ascertaining whether it is active towards the α-alkyl ethers of the hexoses or towards the stereo-isomeric β-ethers. E. F. Armstrong* has extended Fischer's work in this direction by showing that when the α-alkyl ethers of the hexoses are decomposed hydrolytically by enzymes of the maltase type the sugars immediately liberated are the α-forms of the hexoses, and that, similarly, when the stereo-isomeric β-ethers are decomposed by enzymes, they furnish the β-forms of the hexoses.

In both cases the forms of the hexoses first liberated in the solution change, gradually if left to themselves, or immediately if a trace of alkali be added to the liquid, producing the equilibrium mixtures of the α- and β-forms of the hexoses, thus exhibiting the phenomenon of mutarotation which Lowry† first explained in this way.

E. F. Armstrong‡ has extended these results to a number of the naturally occurring sugars and also to several of the synthetic glucosides, and has succeeded in showing that by the action of invertase on sucrose and raffinose the α-form of dextrose is produced and that emulsin decomposes β-methyl glucoside, liberating the β-form of dextrose.

Action of Yeast on Phaseolunatin.

Since it had proved impossible to decompose phaseolunatin by emulsin, which is an enzyme of the β-class, it seemed advisable to ascertain if it could be decomposed by yeast-maltase. The latter was prepared by mixing yeast, previously dried at 30° C., with sand and grinding the mixture with a small quantity of water in a mortar. The paste so obtained was set aside with more water for 24 hours and maintained at a temperature of 35° C., 1 per cent. of toluene being added as an antiseptic.

The aqueous extract so obtained was filtered through a porous tube of baked clay, and by this means a pale straw-coloured liquid was obtained free from yeast cells. This liquid readily decomposed α-methyl glucoside and phaseolunatin. The results of several of the experiments made are given in the following table:—

* 'Chem. Soc. Journ.,' 1903, vol. 83, p. 1305.
† 'Chem. Soc. Journ.,' 1899, vol. 75, p. 213.
‡ *Loc. cit.*

Name of glucoside used.	Amount of maltase preparation used.	Time of action.	Amount of glucoside decomposed.
	c.c.	hours.	per cent.
A. α-Methyl glucoside, 0·2 gramme	20	25	59·0*
Phaseolunatin, 0·3 gramme	20	25	30·5†
B. Phaseolunatin, 0·3 gramme	30	20	22·5†
„ 0·3 gramme	30	30	30·0†
α-Methyl glucoside, 0·2 gramme	10	20	26·0*

* Determined by estimating the dextrose produced.
† „ „ „ prussic acid produced.

The action was found to proceed more readily, and could be more easily followed when, as suggested by Fischer,* dried yeast was substituted for the extract. Thus, in one particular case, using yeast dried at 30° C., 85 per cent. of the phaseolunatin was decomposed in 24 hours.

These results indicate that phaseolunatin is an α-glucoside, since it is decomposed by yeast-maltase. Attempts were therefore made to ascertain by experiment whether α-dextrose is produced initially when the glucoside is hydrolysed by an enzyme.

Nature of the Dextrose Residue in Phaseolunatin.

A preparation of the enzymes contained in the beans of *Phaseolus lunatus* was made by extracting the beans with cold water, exposing the extract in a vacuous desiccator over sticks of potassium hydroxide to remove the hydrocyanic acid formed and then pouring the aqueous extract into excess of alcohol. The precipitate so obtained was dried on a porous tile at the atmospheric temperature.

The characterisation of the sugar produced by the decomposition of phaseolunatin was carried out as follows. To a solution of 3 grammes of the glucoside in 20 c.c. of water, 0·5 gramme of the enzyme preparation was added and the mixture kept at 40° C. in a stoppered bottle.

For each observation a few cubic centimetres of the liquid were withdrawn, mixed with a small definite quantity of alumina cream and immediately filtered through a closely felted pad of asbestos. The optical rotation of the clear filtrate was then determined *before* and *after* the addition of a drop of ammonia solution, the latter being used as indicated by Lowry† to establish equilibrium. The same polarimeter tube was used throughout the experiments.

* 'Berichte,' 1894, vol. 27, p. 1479.
† 'Chem. Soc. Journ.,' 1899, vol. 75, p. 213.

Series 1.—Temperature, 39° C.

Time of action.	Initial rotation.	Rotation after addition of ammonia.	Change in rotation.
hours.	° ′	° ′	° ′
0	−1 15	−1 15*	0 0
1	−0 46	−1 11	−0 25
2	−0 35	−0 58	−0 23
3	−0 34	−0 57	−0 23
20	−0 56	−0 56	0 0

* The non-occurrence of any immediate change in rotation indicates that the optical rotation of phaseolunatin is not affected by the addition of the minute quantity of alkali necessary in carrying out these experiments.

In this series of experiments 2·85 grammes of phaseolunatin were dissolved in 20 c.c. of water, and to the solution 0·5 gramme of the enzyme preparation was added.

Series 2.—Temperature, 40° C.

Time of action.	Initial rotation.	Rotation after addition of ammonia.	Change in rotation.
hours.	° ′	° ′	° ′
0·0	−1 0	−1 0	0 0
0·5	−0 44	−0 59	−0 15
1·5	−0 38	−0 55	−0 17
3·0	−0 29	−0 50	−0 21
20·0	−0 50	−0 50	0 0

The various portions of the liquid resulting from these two series of experiments were mixed and boiled with dilute sulphuric acid to ensure complete decomposition of the glucoside. The acid liquid was then neutralised with barium carbonate, evaporated to dryness on the water bath, and the residue extracted with strong alcohol. This extract was then decolourised with animal charcoal, and the contained sugar precipitated by adding excess of ether. The sticky precipitate was dissolved in diluted alcohol, from which on standing the sugar separated in the form of minute colourless plates, melting at 85°, which is the melting point of dextrose. It had, moreover, the same specific rotation as dextrose. It was shown in the previous paper* that the sugar produced by the hydrolysis of phaseolunatin is dextro-rotatory, and yields a phenylglucosazone, melting at 208°. The sugar produced by the hydrolysis of phaseolunatin is therefore dextrose.

* Dunstan and Henry, 'Roy. Soc. Proc.,' 1903, vol. 72, p. 285.

Further, the tabulated results given above show that an increase in the lævo-rotatory power of a solution of phaseolunatin, partially hydrolysed by the natural enzyme, is produced by the addition of ammonia. This can only mean that a highly dextro-rotatory substance initially produced by the action of the enzyme is transformed by the ammonia into one of lower dextro-rotatory power.

It will be seen that, taking the following data into account,

Specific rotation of phaseolunatin		.....	$-27°{\cdot}4$
„	„	α-dextrose	$+105°$
„	„	β-dextrose	$+22°$
„	„	the equilibrium mixture of $\alpha+\beta$-dextrose	$+52°{\cdot}5$

these results can only be explained on the assumption that the sugar initially produced by the decomposition of phaseolunatin is α-dextrose, and that the glucoside itself is the α-dextrose ether of acetonecyanohydrin. Phaseolunatin appears to be the only naturally occurring α-glucoside so far known, all the other glucosides so far examined yielding the β-forms of the sugars on complete hydrolysis by enzymes.

Nature of the Glucosidolytic Enzymes present in Phaseolus lunatus.

Since α-dextrose is produced from phaseolunatin by the enzyme preparation obtained as already described from the beans of *Phaseolus lunatus*, it must be assumed that this preparation contains an α-enzyme and, since the glucoside is also decomposed by the maltase of yeast, it would appear that this α-enzyme may be identical with yeast maltase, or is at any rate of the same type.

The only other possible view is that the α-enzyme of *Phaseolus lunatus* may be identical with the invertase of yeast, but Fischer has shown that the latter does not decompose α-methyl glucoside, and we have found that yeast washings, containing invertase, have no action on phaseolunatin. In these two respects, therefore, invertase differs from the α-enzyme of *Phaseolus lunatus*.

The identity of the α-enzyme of *Phaseolus lunatus* with yeast maltase cannot, however, be definitely established, since, although the range of activity of the two enzymes appears to be similar, yeast maltase decomposes α-methyl glucoside and maltose more rapidly than does the α-enzyme of *Phaseolus lunatus*, and the latter hydrolyses phaseolunatin more quickly than yeast maltase does.

Since the enzyme preparation obtained from *Phaseolus lunatus* decomposes

amygdalin and salicin, and in this respect resembles the emulsin of almonds, it must also contain a β-enzyme identical with or similar to emulsin. The only other possible hypothesis, viz., that *Phaseolus lunatus* contains a single glucosidolytic enzyme capable of decomposing both α- and β-glucosides, seems incompatible with our present views of the relationships of enzymes and glucosides.

A mixture of the same two enzymes must also occur in cassava and in flax* since, as we have shown previously, enzyme preparations obtained from these two sources behave in precisely the same manner as the enzyme preparation obtained from *Phaseolus lunatus*. It is also worthy of mention in this connection that yeast is capable of decomposing phaseolunatin, amygdalin, and salicin, and on this and other grounds has been assumed to contain an emulsin-like enzyme as well as maltase.†

* Dunstan, Henry, and Auld, 'Roy. Soc. Proc.,' 1905, vol. 78, p. 145.

† Henry and Auld, 'Roy. Soc. Proc.,' 1905, vol. 76, p. 568.